Immune complex-induced enhancement of bacterial antigen presentation requires Fcγ Receptor III expression on dendritic cells

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Dendritic cells (DCs) are capable of initiating adaptive immune responses against infectious agents by presenting pathogen-derived antigens on MHC molecules to naïve T cells. Because of their key role in priming adaptive immunity, it is expected that interfering with DC function would be advantageous to the pathogen. We have previously shown that *Salmonella enterica* serovar Typhimurium (ST), is able to survive inside DCs and interfere with their function by avoiding activation of bacteria-specific T cells. In contrast, when ST is targeted to Fcγ receptors on the DC surface, bacteria are degraded and their antigens presented to T cells. However, the specific Fcγ receptor responsible of restoring presentation of antigens remains unknown. Here, we show that IgG-coated ST was targeted to lysosomes and degraded and its antigens presented on MHC molecules only when the low-affinity activating FcγRIII was expressed on DCs. FcγRIII-mediated enhancement of Ag presentation led to a robust activation of T cells specific for bacteria-expressed antigens. Laser confocal and electron microscopy analyses revealed that IgG-coated ST was rerouted to the lysosomal pathway through an FcγRIII-dependent mechanism. PI-3K activity was required for this process, because specific inhibitors promoted the survival of IgG-coated ST inside DCs and prevented DCs from activating bacteria-specific T cells. Our data suggest that the DC capacity to efficiently activate T cells upon capturing IgG-coated virulent bacteria is mediated by FcγRIII and requires PI-3K activity.

Fcγ receptors | phosphoinositide-3 kinase | *Salmonella* Typhimurium | T cells | antigen-presenting cells

Initiation of adaptive immunity in response to pathogenic bacteria requires that dendritic cells (DCs) residing at the infection site recognize pathogen-associated molecular patterns (PAMPs) (1–3). Upon PAMP recognition, immature DCs undergo a phenotypic change, known as maturation (4, 5), which empowers them to efficiently process and present bacteria-derived antigens on MHC molecules to naïve $CD4^+$ and $CD8^+$ T cells (1, 6). Considering that DCs have the unique capacity to directly prime naïve $CD4^+$ and $CD8^+$ T cells (7), interfering with their function can be highly advantageous for the dissemination and survival of pathogenic bacteria. Several studies have provided evidence supporting the notion that virulent bacteria are capable of interfering with the capacity of DCs to activate T cells (8–12), thereby impairing initiation of adaptive immunity.

Salmonella enterica serovar Typhimurium (herein ST), a Gram-negative bacteria that causes a self-limiting gastroenteritis infection in humans and a typhoid-like systemic disease in mice, can survive within murine DCs and avoid antigen presentation to T cells (10). This feature of pathogenic ST requires expression of specific virulence genes, some of which are encoded by *Salmonella* pathogenicity islands 1 and 2 (SPI-1 and -2) (11, 13–15). Although SPI-1-encoded genes are essential for bacterial-induced internalization in nonphagocytic cells such as the intestinal epithelium (16), SPI-2-encoded genes are necessary for ST survival within phagocytic cells (17) such as macrophages (18) and DCs (11, 15). Some effector proteins translocated to the host cell cytoplasm by the SPI-2-encoded type III secretion system (TTSS) contribute to impair processing of bacterial antigens by preventing the fusion of ST-containing vacuoles (SCV) with lysosomes (11, 19).

DCs can capture extracellular antigens by either pinocytosis or receptor-mediated endocytosis (20–23). However, this latter mechanism has been shown to significantly increase the efficiency of antigen uptake, processing, and presentation on MHC molecules to T cells (21, 22, 24). In this direction, several studies support the notion that Fcγ receptor-mediated uptake of IgG immune complexes (ICs) can considerably enhance the capacity of DCs in processing and presenting antigens to T cells (10, 22, 25–27). Furthermore, we have recently shown that DC uptake of virulent ST by means of FcγRs rerouted bacteria to the lysosomal degradation pathway, which allowed DCs to overcome the capacity of these bacteria to avoid lysosomal fusion and antigen presentation to T cells (10). However, neither the specific FcγR nor the signaling pathway responsible for enhancing the ability of DCs to degrade virulent bacteria has been identified.

Because DCs express both low-affinity FcγRs [activating (FcγRIII) and inhibitory (FcγRIIB)], we evaluated the differential capacity of DCs deficient in individual FcγRs to degrade IgG-coated virulent ST and to process and present bacterial antigens to T cells. Our data suggest that the activating FcγRIII is the main receptor responsible for targeting ST to the lysosomal pathway. Furthermore, we show that, to efficiently degrade and present ST-derived antigens to T cells, FcγRIII requires phosphoinositide-3 kinase (PI-3K) activity in DCs.

Results

Presentation of Bacteria-Expressed Antigens and T Cell Activation Is Observed Only When Virulent ST Is Targeted to FcγRs on DCs. The capacity of ST to evade adaptive immune responses finds support from several recent independent studies (10, 11, 28–30). We and others have reported that virulent strains of ST can efficiently evade antigen presentation on DCs, preventing activation of bacteria-specific T cells (10, 11, 15). Lack of T cell activation by DCs infected with virulent ST strains expressing ovalbumin (ST-pOVA) as a

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Abbreviations: DC, dendritic cell; PAMP, pathogen-associated molecular pattern; SPI, *Salmonella* pathogenicity island; TTSS, type three secretion system; IC, immune complex; PI-3K, phosphoinositide-3 kinase; LAMP-2, lysosomal-associated membrane protein 2; MOI, multiplicity of infection.

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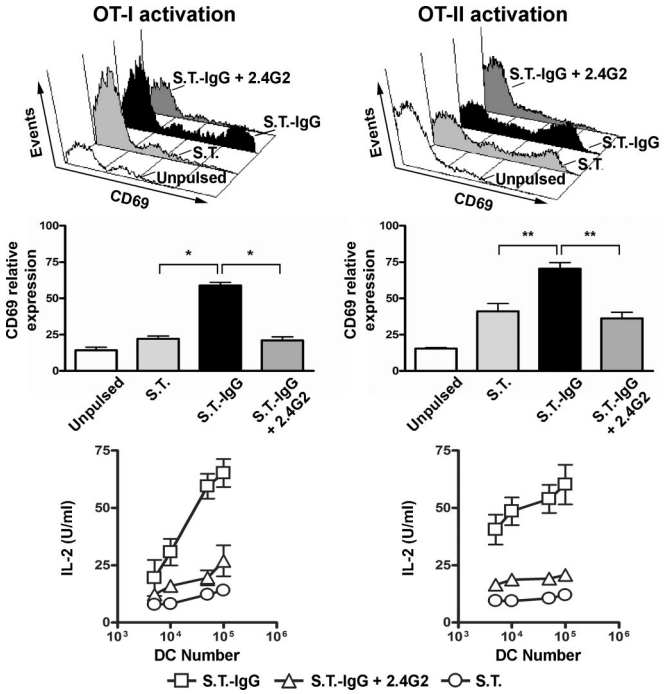

Fig. 1. T cell activation is achieved only when virulent ST is internalized by means of FcγRs on DCs. WT DCs were pulsed with either free or IgG-coated ST-pOVA and cocultured with OT-I (*Left*) or OT-II (*Right*) T cells, as described in *Materials and Methods*. Unpulsed and 2.4G2-treated DCs were included as controls. After 20 h, CD69 expression and IL-2 release by T cells were determined. (*Top*) Representative histograms for CD69 expression in T cells. (*Middle*) Relative CD69 expression (100% = 10 μg/ml OVA protein). (*Bottom*) IL-2 release by T cells. Data are means ± SD of triplicates from at least four independent experiments. *, $P < 0.05$; **, $P < 0.01$ by unpaired Student *t* test.

traceable antigen was demonstrated for OT-I or OT-II transgenic T cells, which recognize OVA-derived peptides loaded on MHC-I and MHC-II molecules, respectively (Fig. 1). Consistent with previous observations (10), OVA-specific T cells failed to up-regulate surface expression of CD69 and did not secrete IL-2 in response to DCs infected with free ST-pOVA (Fig. 1). The absence of T cell activation was not due to a cytotoxic effect on DCs caused by bacteria, because these cells were able to prime T cells when pulsed simultaneously with bacteria and purified OVA protein or antigenic peptides (data not shown).

In contrast, when DCs were pulsed with IgG-coated ST-pOVA, activation of $CD4^+$ and $CD8^+$ T cells was restored, as determined by CD69 up-regulation and IL-2 secretion (Fig. 1). This was the case for ST-pOVA opsonized with either rabbit (Fig. 1) or mouse [supporting information (SI) Fig. 6] IgG. To determine whether restoration of antigen processing and presentation was mediated by low-affinity FcγRs, DCs were treated with 2.4G2, a blocking monoclonal antibody specific for the low-affinity receptors FcγRIIB and FcγRIII before pulsing with IgG-coated ST. We observed that, as a result of simultaneous FcγRIIB and FcγRIII blockade, no T cell activation was induced by DCs pulsed with IgG-coated ST (Fig. 1). These data would suggest that low-affinity FcγRs are mainly responsible for restoring T cell activation when virulent ST is targeted to FcγRs on DCs.

Targeting Virulent ST to FcγRIII Is Required to Restore the Capacity of DCs to Activate Bacteria-Specific T Cells. To define the low-affinity FcγR responsible for promoting presentation of ST-expressed antigens to T cells, we evaluated the capacity of DCs deficient in individual FcγRs to activate T cells in response to IgG-coated bacteria. DCs were obtained from either FcγRIIB- or FcγRIII-deficient mice and pulsed with IgG-coated virulent ST. T cell activation was determined by up-regulation of CD69 expression and IL-2 secretion. As shown in Fig. 2*B*, DCs derived from FcγRIII-deficient mice were unable to activate OT-I or OT-II transgenic T cells in response to IgG-coated ST-pOVA. In contrast, $CD4^+$ and $CD8^+$ T cells were efficiently activated by DCs obtained from FcγRIIB-deficient mice, as shown by significant CD69 up-regulation and IL-2 release (Fig. 2*A*). Equivalent results were obtained when FcγR-deficient DCs were pulsed with ST coated with either rabbit (Fig. 2) or mouse IgG (SI Fig. 7). Further, FcγR-blockade with 2.4G2 mAb prevented T cell activation by FcγRIIB-deficient DCs pulsed with IgG-coated ST (Fig. 2*A*). These results suggest that a significant enhancement of the capacity of DCs to induce an efficient T cell activation is observed only when IgG-opsonized virulent ST is internalized by means of activating FcγRIII. In contrast, targeting IgG-coated ST to the inhibitory FcγRIIb fails to promote T cell activation.

FcγRIII-Deficient DCs Fail to Efficiently Process and Present Antigens Derived from IgG-Coated ST. To test whether a reduced capacity to degrade IgG-coated ST was the mechanism responsible for the inability of FcγRIII-deficient DCs to activate bacteria-specific T cells, we measured ST survival inside DCs by gentamicin protection assays (11). Consistent with previous studies, free ST was able to survive inside DCs at least 12 h after infection (Fig. 3*A*) (10). In contrast, IgG-coated ST showed significantly reduced intracellular survival only in WT and FcγRIIB-deficient DCs, but not in FcγRIII-deficient DCs (Fig. 3*A*). Furthermore, decreased bacterial survival for IgG-coated ST was observed at different time points in WT and FcγRIIB-deficient DCs, but not in FcγRIII-deficient DCs (Fig. 3*B*). These observations suggest that activating FcγRIII is required for degradating IgG-coated ST in DCs.

To evaluate whether the lack of efficient bacterial degradation shown by FcγRIII-deficient DCs could lead to reduced antigen processing and presentation on MHC, the surface density of $H\text{-}2K^b$/SIINFEKL complexes was measured on DCs pulsed with free ST-pOVA or IgG-coated ST-pOVA. Consistent with the intracellular bacteria survival assays, $H\text{-}2K^b$/SIINFEKL complexes were detected only on the surface of WT and FcγRIIB-deficient DCs pulsed with IgG-coated ST (Fig. 3*C*). In contrast, no measurable $H\text{-}2K^b$/SIINFEKL complexes were detected on the surface of FcγRIII-deficient DCs pulsed with IgG-coated ST. In accord with previous studies, $H\text{-}2K^b$/SIINFEKL was not observed for DCs pulsed with free virulent ST (10, 11). These data support the notion that activating FcγRIII is required for targeting IgG-coated ST for degradation and antigen processing pathways on DCs. In contrast, neither significant bacteria degradation nor antigen processing were observed when IgG-coated ST was targeted to the inhibitory FcγRIIb.

FcγRIII Is Required to Direct IgG-Coated Virulent ST to Lysosomal Degradation on DCs. Previous studies have shown that virulent ST can survive inside DC vacuoles, probably by avoiding fusion with lysosomes (10, 11, 19). In contrast, IgG-coated ST is targeted to lysosomal degradation in a FcγR-dependent fashion (10). To identify the low-affinity FcγR involved in this process, we used confocal microscopy to determine the capacity of FcγRIIB- and FcγRIII-deficient DCs to target IgG-coated ST-pGFP to vacuoles containing lysosomal markers, such as LAMP-2. As shown in Fig. 4, IgG-coated ST significantly colocalized with LAMP-2-containing vesicles only in WT and FcγRIIB-deficient DCs (85.1% and 85.7% colocalization for WT and $FcγRIIB^{-/-}$ DCs, respectively, Fig. 4 *B*, *D*, and *G*). In contrast, IgG-coated ST and LAMP-2 colocalization was significantly reduced (17% colocalization) for FcγRIII-deficient DCs (Fig. 4 *F* and *G*). Consistent with previous studies (10, 11), no significant colocalization between ST and LAMP-2 was observed for any of the DCs tested when pulsed with

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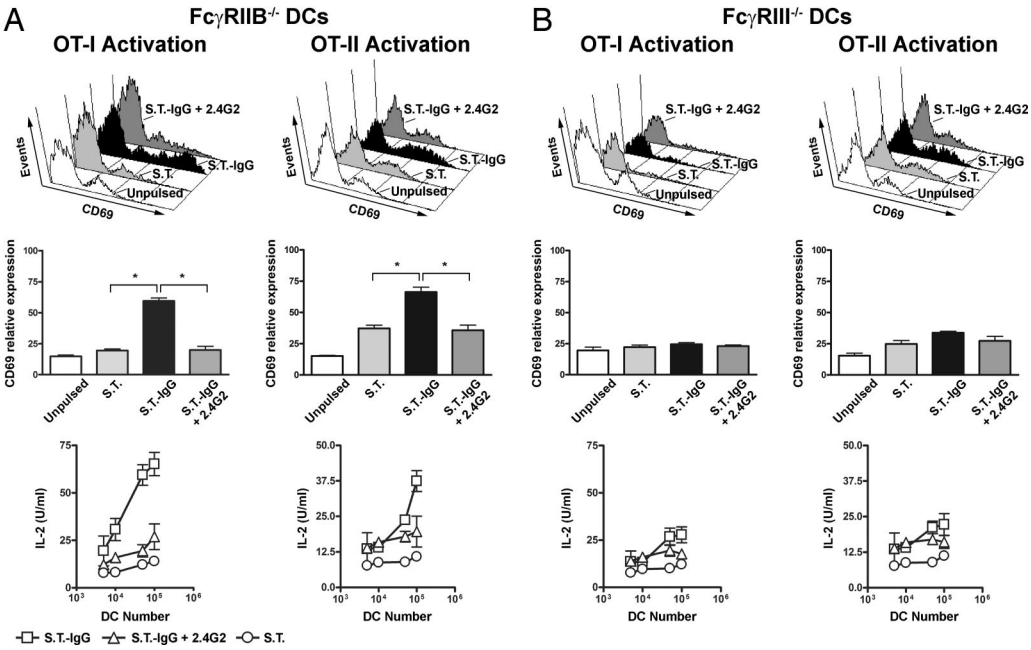

Fig. 2. FcγRIII expression on DCs is required to activate T cells in response to IgG-coated virulent ST. FcγRIIB$^{-/-}$ (*A*) or FcγRIII$^{-/-}$ (*B*) DCs were pulsed with either free or IgG-coated ST-pOVA and cocultured with OT-I (*Left*) or OT-II (*Right*) T cells, as described in *Materials and Methods*. Unpulsed and 2.4G2-treated DCs were included as controls. After 20 h, CD69 expression and IL-2 release by T cells were determined. (*Top*) Representative histograms for CD69 expression in T cells. (*Middle*) Relative CD69 expression (100% = 10 μg/ml OVA protein). (*Bottom*) IL-2 release by T cells. Data are means ± SD of triplicates from at least four independent experiments. *, $P < 0.01$ by unpaired Student *t* test.

free bacteria (15.5%, 14.4%, and 14.3% for FcγRIII$^{-/-}$, WT, and FcγRIIB$^{-/-}$ DCs, respectively) (Fig. 4 *A*, *C*, *E*, and *G*).

In agreement with the confocal microscopy data, electron-light vesicles in the cytoplasm of intracellular bacteria evidencing degradation (31) were observed only in electron micrographs of WT and FcγRIIB-deficient DCs pulsed with IgG-coated ST (SI Fig. 8 *B*, *D*, and *G*). In contrast, no such electron-light structures were observed in bacteria contained in the vacuoles of FcγRIII-deficient DCs pulsed with ST-IgG (SI Fig. 8 *F* and *G*). These observations support the notion that the activating FcγRIII is required for targeting IgG-opsonized virulent ST to the lysosomal degradation pathway. Consistent with our previously published results (10, 11), no signs of bacterial degradation were observed in any of the DC types after infection with free virulent ST (SI Fig. 8 *A*, *C*, *E*, and *G*).

Degradation of ST Internalized by Means of FcγR on DCs Requires PI-3K Activity. Next, we tested whether the ability of DCs to degrade FcγR-captured ST was the result of an impaired capacity of ST to translocate virulence factors to the DC cytoplasm caused by IgG opsonization or, alternatively, by an FcγRs-triggered molecular mechanism. By confocal microscopy, we analyzed translocation of SseB, a SPI-2-codified effector protein, to the DC cytoplasm. SseB, together with SseC and SseD, is secreted by vacuole-residing ST and compose a TTSS that translocates bacteria effector proteins to the host cell cytoplasm (32, 33). We observed that SseB was secreted to the DC cytoplasm by ST, even when bacteria were opsonized with IgG (data not shown). Consistent with this finding, equivalent amounts of SseB were secreted by both free and IgG-coated ST grown in N medium, which mimics phagosome environment (data not shown). These results suggest that ST opsonization with IgG did not significantly impair the capacity of bacteria to translocate effector molecules into the DC cytoplasm.

Considering that the capacity of IgG-coated ST to translocate virulence factors seemed intact, we evaluated whether enhanced bacterial degradation observed upon FcγRIII uptake of IgG-coated ST could be mediated directly by FcγR signaling. Considering that PI-3K is a key element involved in FcγRIII signaling and has been implicated in phagosome maturation (34, 35), the activity of this enzyme was inhibited on ST-infected DCs by treating with wortmannin 30 min after bacterial infection (36). These experimental conditions did not impair bacterial entrance to DCs, as shown by gentamicin protection assays (data not shown). As shown in Fig. 5, wortmannin significantly enhanced the survival of IgG-coated ST inside WT DCs (Fig. 5*A*). Consistent with these observations, wortmannin treatment of DCs pulsed with IgG-coated ST significantly impaired their capacity to activate T cells (Fig. 5*B*). An equivalent inhibition of T cell activation was observed on DCs pulsed with either rabbit- or mouse-IgG-coated ST-pOVA and treated with wortmannin (SI Fig. 6). In contrast, wortmannin did not prevent T cell activation induced by DCs pulsed with either OVA protein or OVA antigenic peptides (data not shown). These data suggest that inhibition of PI-3K on DCs impaired only their capacity to prime T cells when pulsed with IgG-coated ST-pOVA but not with soluble OVA. These results support the notion that PI-3K activity is necessary for normal phagosome maturation by means of FcγRIII in DCs as well as for presentation of antigens derived from IgG-opsonized bacteria to T cells.

Discussion

We had previously shown that the capacity of virulent ST to interfere with the ability of DCs to present antigens and activate T cells can be overcome by targeting bacteria to FcγRs on these cells (10). Here, we expand on this knowledge by providing functional evidence supporting the notion that the activating FcγRIII is the receptor responsible for targeting IgG-opsonized ST to the lysosomal degradation pathway in DCs. We show that internalization by means of FcγRIII is required to restore the capacity of DCs to present bacteria-expressed antigens on MHC molecules and activate T cells in response to IgG-coated virulent ST.

Virulent ST have developed different molecular strategies to evade host adaptive immunity, such as induction of caspase-1-dependent apoptosis in macrophages and DCs (37) as well as a

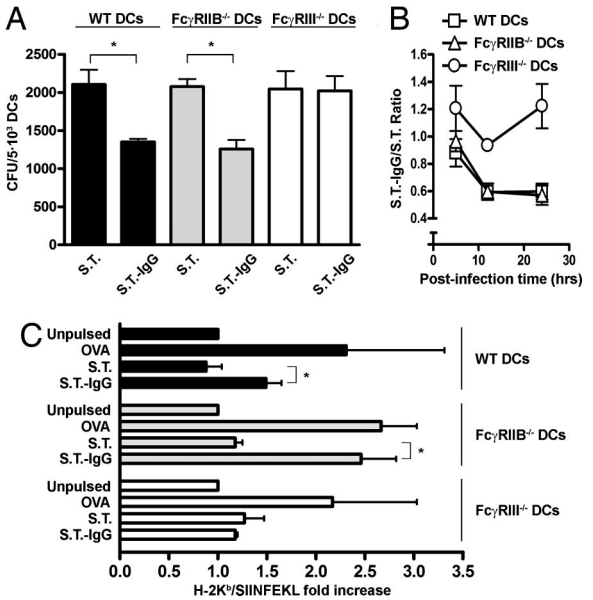

Fig. 3. FcγRIII-mediated internalization of IgG-coated ST promotes bacterial degradation and antigen presentation to T cells by DCs. WT, FcγRIIB$^{-/-}$ or FcγRIII$^{-/-}$ DCs were pulsed with either free or IgG-coated ST, and survival of intracellular bacteria was determined by gentamicin protection assays. (*A*) Intracellular ST survival for WT (black bars), FcγRIIB$^{-/-}$ (gray bars), and FcγRIII$^{-/-}$ (white bars) DCs at 12 h after infection. (*B*) Intracellular survival ratio between IgG-coated and free ST in WT (squares), FcγRIIB$^{-/-}$ (triangles), and FcγRIII$^{-/-}$ (circles) DCs at 5, 12, or 24 h after infection. (*C*) Antigen presentation was evaluated by detecting H-2K^b/SIINFEKL complexes on the surface of DCs (WT, black bars; FcγRIIB$^{-/-}$, gray bars; FcγRIII$^{-/-}$, white bars) challenged with free or IgG-coated ST-pOVA. As controls, unpulsed DCs (Unpulsed) or DCs pulsed with 10 μg/ml OVA (OVA), were included. H-2K^b/SIINFEKL expression data are the fold increase relative to expression by unpulsed DCs. Data shown are means ± SD from three independent experiments. *, $P < 0.05$ by unpaired Student *t* test.

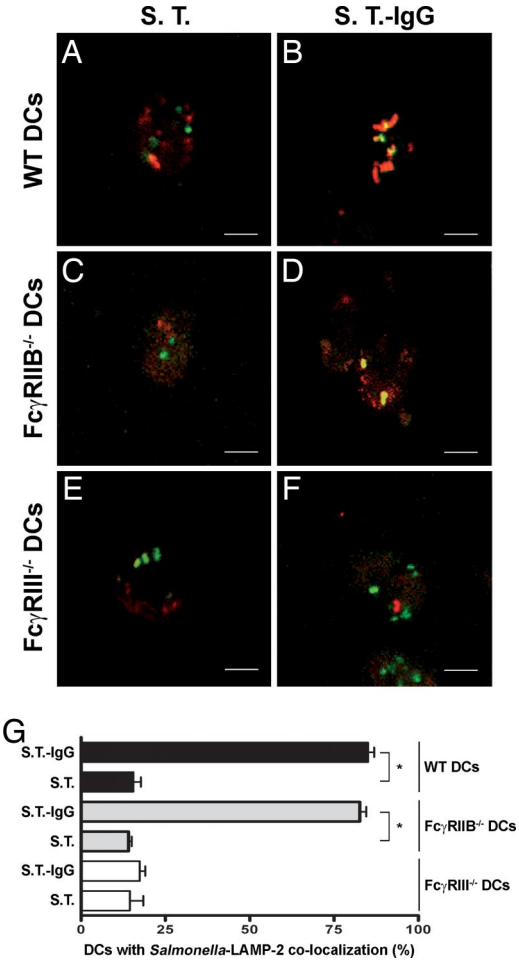

Fig. 4. FcγRIII on DCs reroutes virulent ST to the lysosomal degradation pathway. WT, FcγRIIB$^{-/-}$ or FcγRIII$^{-/-}$ DCs were pulsed with either free or IgG-coated ST-pGFP and stained for LAMP-2. GFP (green) LAMP-2 (red) colocalization (yellow) was analyzed by confocal microscopy. (*A–F*) Representative merged images from WT (*A* and *B*), FcγRIIB$^{-/-}$ (*C* and *D*), or FcγRIII$^{-/-}$ (*E* and *F*) DCs infected with either free (*Left*) or IgG-coated ST-pGFP (*Right*) are shown. (Scale bars, 5 μm.) (*G*) Quantitative analyses for GFP-LAMP-2 colocalization were performed by using the LSM 5 Examiner software (Zeiss). Data shown are means ± SD of three independent experiments. *, $P < 0.05$ by unpaired Student *t* test.

direct inhibition of T cell activation by a contact-dependent mechanism (10–12, 38). Considering that DCs have the unique capacity to capture bacteria at the site of infection and efficiently process and present bacterial antigens to prime naïve T cells (1, 6, 39), it is likely that interference with DC function would promote bacterial dissemination. This notion is consistent with the observation that virulent strains of ST are capable of surviving inside DCs, avoiding antigen presentation to T cells (10, 11, 19). This feature requires functional expression of SPI-2-encoded effector proteins, because ST strains deficient in SPI-2 genes fail to survive inside DCs and are degraded and their antigens presented to bacteria-specific T cells (11).

Although virulent ST is able to survive inside DCs and avoid antigen presentation, the bacterium is degraded and its antigens presented to T cells when internalized by means of FcγRs on DCs (10). Here, we show that FcγRIII is the receptor responsible for restoring the capacity of DCs to degrade bacteria and present antigens to T cells, when challenged by IgG-coated ST. FcγRIII-mediated uptake targeted IgG-coated ST to the lysosomal pathway, which increased the efficiency of presentation of bacteria-derived antigens on MHC molecules. Consistently, challenge with IgG-opsonized ST led to an increase in the amount of MHC molecules loaded with bacteria-derived antigenic peptides on WT and FcγRIIB$^{-/-}$ DCs, which empowered them for activating T cells. This was not the case for FcγRIII-deficient DCs, suggesting that this receptor is required for the degradation of IgG-opsonized ST and the subsequent presentation of bacterial antigens in MHC molecules on DCs. Furthermore, the observation that IgG-coated ST were not targeted to the lysosomal degradation pathway in FcγRIII-deficient DCs suggests that internalization by means of FcγRIII is required to reroute virulent ST to lysosomal degradation and promote antigen presentation. Confocal analyses of ST-infected DCs and protein secretion assays on bacteria indicated that neither IgG opsonization nor FcγRIII-mediated internalization interfered with the capacity of virulent ST to translocate SPI-2-encoded effector molecules. This notion was further supported by the observation that IgG-coated ST is able to survive and even replicate inside FcγRIII$^{-/-}$ DCs. According to these findings, it is likely that enhanced bacterial degradation and antigen presentation of IgG-coated ST is promoted by mechanisms involving directly the signaling through FcγRIII. Engagement of FcγRIII by ICs involves activation of PI-3K activity, among other signaling molecules (40, 41). It has been shown that PI3K contributes to phagosomal maturation through recruitment of key molecules, such as EEA1, Rab5, and Rab7 (42–44). Although class I PI-3K is involved in some of the early events required to form the phagosomal vacuole, class III PI-3K is required for vacuole-lysosome fusion (42, 43). To promote inhibition of class III PI-3K-dependent lysosomal fusion without affecting class I PI-3K-dependent bacteria uptake, we blocked PI-3K activity 30 min after pulsing with IgG-coated ST. PI-3K inhibition led to an impairment in bacterial degradation and

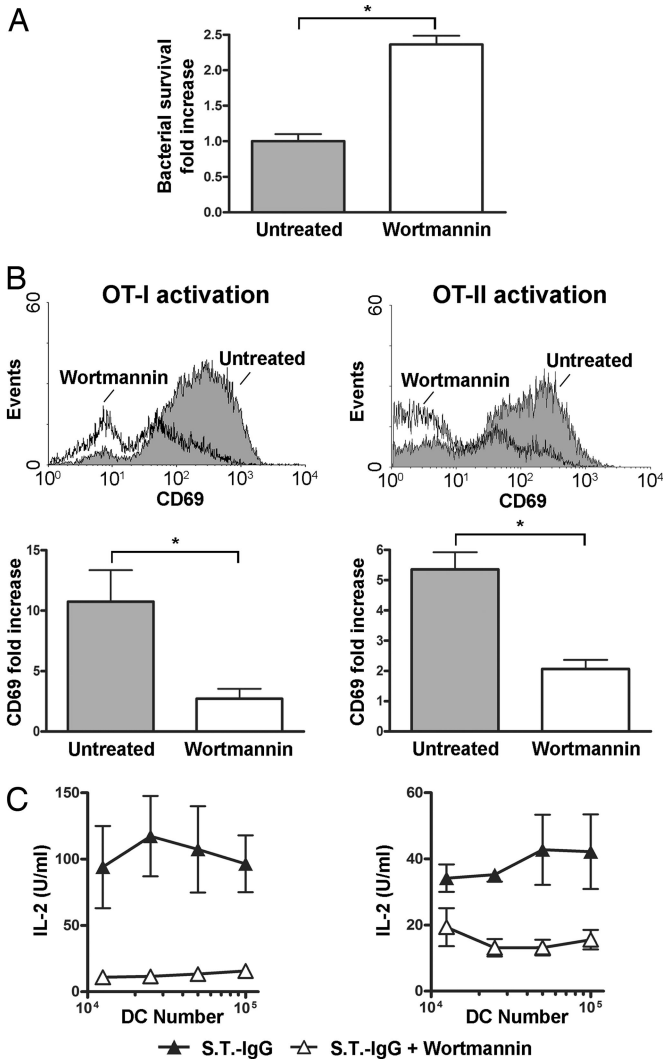

Fig. 5. PI-3K activity on DCs is required for FcγRIII-mediated bacterial degradation and T cell activation. (*A*) WT DCs were pulsed with IgG-coated ST and then treated with 100 nM wortmannin, and survival of intracellular bacteria was determined by gentamicin protection assays. (*B* and *C*) CD69 up-regulation (*B*) and IL-2 release (*C*) by OT-I and OT-II T cells in response to DCs pulsed with IgG-coated ST and treated with wortmannin. Histograms are representative, and data shown are means ± SD of three independent experiments. *, $P < 0.05$ by unpaired Student *t* test.

T cell activation by DCs pulsed with IgG-coated ST, suggesting that PI-3K activity is required for FcγRIII-mediated enhancement of ST degradation and presentation of bacterial antigens to T cells. However, further studies are required to identify all of the molecular components involved in the signaling pathways triggered by engagement of FcγRIII by IgG-coated bacteria that could be responsible for enhancing antigen processing by DCs.

The role of FcγRIII as an enhancer of the DC capacity to present bacterial antigens to T cells and initiate adaptive immunity is consistent with previous reports showing that FcγRs can contribute to protective immunity against pathogens, such as *Streptococcus pneumoniae* and *Leishmania* (45, 46). Our data provide an explanation for the involvement of FcγRs on the activation of T cell immunity, which is consistent with the dual requirement of T cells and antibodies for protection against virulent ST strains (47, 48). Thus, although virulent ST is able to survive inside DCs and evade antigen presentation to T cells, IgG-opsonized ST would be degraded by an FcγRIII-mediated mechanism and, subsequently, its antigens presented on the surface of DCs to bacteria-specific T cells, which could contribute to reduce bacterial dissemination.

Materials and Methods

Mice. WT C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). FcγRIIB$^{-/-}$ and FcγRIII$^{-/-}$ mice (C57BL/6 background) were generously provided by T. Takai (Tohoku University, Tohoku, Japan) and K. Smith (University of Cambridge, Cambridge, U.K.), respectively. OT-I and OT-II transgenic mouse strains expressing specific TCR for H-2K^b/OVA$_{257–264}$ and I-A^b/OVA$_{323–337}$, respectively, were obtained from R. Steinman (The Rockefeller University, New York, NY). All mice were maintained and manipulated according to institutional guidelines at the specific pathogen-free facility of the Pontificia Universidad Católica de Chile.

Bacterial Strains. Virulent ST (14028s; American Type Culture Collection, Manassas, VA) was provided by G. Mora (Universidad Andrés Bello, Santiago, Chile). OVA- and GFP-expressing ST were generated as described (10). Bacteria were grown overnight in LB broth at 37°C, and recombinant bacteria were selected by using 50 μg/ml carbenicillin (for OVA-expressing ST) or 100 μg/ml ampicillin (for GFP-expressing ST). For infection of DCs, overnight bacterial cultures were 1/100 diluted and grown until exponential phase (OD_{600} = 0.5–0.7). Mouse ST antiserum was produced by immunizing C57BL/6 mice i.p. with heat-killed-ST (10^4 CFU) emulsified in Freund's complete adjuvant (Sigma–Aldrich, St. Louis, MO). Boosters were given at days 7 and 14 with an equivalent dose of bacteria in Freund's incomplete adjuvant. Antiserum was collected 2 weeks later, and anti-ST titers were determined by ELISA. For generation of IgG-ICs, rabbit anti-ST IgG (0.5 mg/ml Denka–Seiken, Tokyo, Japan), mouse monoclonal anti-ST IgG (1 μg/ml, clone 1E6, isotype IgG1; Advanced Immunochemical, Long Beach, CA) or mouse anti-ST immune sera (30 mg/ml of total protein) were added and incubated for 2–3 h at 4°C. Multiplicity of infection (MOI) and viability of bacterial ICs were confirmed by serial dilutions on LB-agar plates containing the appropriate antibiotic for selection.

T Cell Activation Assays. Bone marrow-derived DCs from C57/BL6, FcγRIII$^{-/-}$ and FcγRIIB$^{-/-}$ mice were prepared as described (10, 22, 49). Briefly, DCs were grown in RPMI medium 1640 with 5% FBS, supplemented with 3% supernatant from J558L cells transduced with murine GM-CSF (49). DCs were pulsed with either free or IgG-coated ST expressing OVA at MOI = 25 during 2 h. Bacteria-pulsed DCs were washed three times with PBS and treated with 50 μg/ml gentamicin to kill extracellular bacteria, as described (10, 50, 51). DC viability was evaluated 12 h later by trypan blue exclusion, and cells were cocultured during 20 h with 1×10^5 OT-I or OT-II T cells. CD69 expression in T cells was determined by FACS by staining with a phycoerythrin-conjugated anti-CD69 (clone H1.2F3; BD Pharmingen, San Diego, CA) and FITC-conjugated anti-CD4 (clone H129.19; BD Pharmingen) or FITC-conjugated anti-CD8 mAb (clone 53–6.7, BD Pharmingen). Secretion of IL-2 was determined by ELISA as described (10, 52–54). In some assays, FcγRIII and FcγRIIB were blocked by incubating with 10 ng/ml of 2.4G2 mAb (BD Pharmingen) during 4 h before pulsing DCs with IgG-coated ST-pOVA. For PI-3K blockade, 100 nM wortmannin (Sigma) was added to DCs 30 min after bacteria challenge. To control bacteria uptake, DCs were lysed after treatment and plated overnight on LB agar for colony count.

Assessment of H-2K^b/SIINFEKL-Complex Assembly on the DC Surface. To determine H-2K^b/SIINFEKL density on the surface of DCs pulsed with either free or IgG-coated ST-pOVA (MOI = 25), cells were stained with phycoetythrin-conjugated anti-CD11c (clone HL3; BD Pharmingen) and supernatant from the 25-D1.16 hybridoma (κ-IgG1 mAb, specific for H-2K^b/SIINFEKL complex; pro-

vided by R. N. Germain, National Institutes of Health, Bethesda, MD) (55) for 1 h at 4°C. DCs were washed with PBS and stained with polyclonal FITC-conjugated goat anti-mouse IgG (BD Pharmingen). The FITC-conjugated goat anti-mouse did not recognize PE-conjugated hamster anti-CD11c antibody (data not shown). DCs were washed with PBS and analyzed by FACS. Data were analyzed by using WinMDI software (downloaded from http://facs.scripps.edu).

Gentamicin Protection Assays. DCs pulsed with either free or IgG-coated ST for 1 h (MOI = 25) were washed with PBS and treated with 50 μg/ml gentamicin for different time intervals. Viable DCs were washed with PBS and lysed by adding 0.5% Triton X-100 in PBS for 30 min. Numbers of viable bacteria released from DCs were assessed by titration on agar plates, as described (10, 11).

Laser Confocal Microscopy. DCs were seeded on cover slides at day 4 of differentiation with GM-CSF. At day 5, DCs were pulsed during 1 h with either free or IgG-coated ST-pGFP at a MOI equal to 25, washed with PBS and treated with 50 μg/ml gentamicin for 3 h. After washing, DCs were fixed with ice-cold methanol at −20°C for 10 min and then blocked with PBS–BSA 3% overnight. Lysosomes were detected by staining DCs with purified rabbit IgG specific for mouse lysosome-associated membrane protein 2 (LAMP-2; Zymed, San Francisco, CA) for 30 min at 4°C. After washing, DCs were stained with Texas red-conjugated anti-rabbit mouse IgG (Zymed) for 45 min at 4°C. DCs were analyzed on an LSM 5 Pascal confocal microscope (Zeiss, Thornwood, NY). Fluorescence extension was plotted by using LSM 5 image examiner software, and semiquantitative analysis was performed by counting the number of DCs exhibiting ST-LAMP-2 colocalization on randomly selected fields.

Electron Microscopy. DCs pulsed with either free or IgG-coated ST at a MOI equal to 25 during 1 h were washed with PBS and treated with 50 μg/ml gentamicin for 3 h. After washing, DCs were fixed overnight in PLP (4% paraformaldehyde, 10 mM periodate, and 200 mM L-lysine on 100 mM phosphate buffer, pH 7.4). Next, DCs were washed with distilled water and incubated in 1% osmium tetroxide at 4°C for 30 min, dehydrated in ethanol and acetone, and embedded in Epon. Thin sections were cut with an OmU2 ultramicrotome (Reichert, Vienna, Austria) and observed under a Tecnai 21 electron microscope (Philips, Eindhoven, The Netherlands).

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